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STUDIES IN A RAT LUNG TUMOR MODEL: CELLULAR BIOCHEMISTRY AND CYTOGENETICS

RONALD E. RASMUSSEN, PH.D.

UNIVERSITY OF CALIFORNIA, IRVINE
COMMUNITY AND ENVIRONMENTAL MEDICINE
IRVINE, CALIFORNIA 92717

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

Roger C. Inman

ROGER C. INMAN, Colonel, USAF
Chief
Toxic Hazards Division
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Dose-response relationships were established for the induction of 3-methylcholanthrene (MCA) of squamous cell carcinomas (SCC) in rat lung. Five biweekly intratracheal inoculations of 0.25, 0.5, 1.0, or 5.0 mg MCA gave tumor yields of 8%, 16%, 48%, and 100%, respectively, in treatment groups of 25 rats each. No tumors were seen in control rats or in rats treated 5 times with 0.1 mg MCA.

Studies of sister chromatid exchange (SCE) incidence in lung cell cultures and in peripheral lymphocytes of rats treated intratracheally with tumorigenic doses of MCA showed elevated frequencies of SCEs in the lung cells, but not in

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peripheral lymphocytes. The increased level of SCEs in the lung cells did not persist, but declined in parallel with the clearance of MCA from the lungs over a period of about 6-8 weeks. In vitro exposure of lung cells from untreated rats to MCA resulted in an increase in the SCE frequency in the cells. As little as 1 μg MCA/ml in the culture medium was effective. The conclusions were that SCE incidence in peripheral lymphocytes could not be used to predict a carcinogenic hit in the lung as the result of MCA treatment, and that elevation of SCE frequency in lung cells was dependent on the presence of MCA immediately prior to performing the assay. No evidence was found for the persistence of lung cell DNA damage which could lead to an increase in SCE frequency.

Carcinogen metabolizing enzymes in the lung and liver were assayed in vitro using microsomal preparations from these tissues and ^{3}H-benzo(a)pyrene (^{3}H-BaP) as substrate. Intratracheal treatment with MCA induced enzyme activity in both lung and liver. The level of the increased activity in the lung paralleled the clearance of MCA from the lung, suggesting that continued presence of MCA was required for enzyme induction. Induced enzyme levels in the liver declined more rapidly than in the lung, and had returned to control levels at times when lung enzymes were still well above controls. This result suggests that xenobiotics reaching the lung via the respiratory tract may induce enzymatic activity there, and undergo substantial metabolism before entering the systemic circulation. Preliminary studies with isolated type II alveolar cells, macrophages, and a preparation enriched in bronchiolar epithelial cells indicated that enzyme activity could be induced in both type II cells and epithelial cells by in vivo treatment with a known inducer of the mixed function oxidases, beta-naphthoflavone.

Reports that naphthalene (N) was specifically toxic to nonciliated bronchiolar cells in the mouse lung served as the basis for a comparative study of the cytotoxic effects of nitronaphthalenes. 1-Nitronaphthalene (1-NN) was about twice as toxic as N in the mouse lung, but 2-NN was much less toxic. Of most interest was the observation that 2-NN induced mixed function oxidase activity specifically in the lung, while the other compounds had no such effect, either in the lung or liver. This lung-specific enzyme induction by 2-NN is unusual, since most xenobiotics induce activity primarily in the liver, and secondarily in other organs. That 2-NN was metabolized in the liver was indicated by the fact that periportal necrosis was seen at a dose (2 mmoles/kg bw ip) that induced enzyme activity in the lung.

Preliminary results of studies in which rats were treated with MCA and/or asbestos indicated that cytotoxic effects in the lung were different for the 2 materials. MCA tended to produce lesions at the terminal bronchioles and asbestos caused focal areas of fibrosis. Experiments in progress will indicate whether a cocarcinogenic action of the materials may occur in the rat lung tumor model.

PREFACE

This is the third annual report of the Cytology, Cell Biology, and Cytogenetics section of the Toxic Hazards Research Program performed by the Department of Community and Environmental Medicine of the University of California, Irvine (UCI) on behalf of the Air Force under contract number F33615-80-C-0512. This report describes the research activities at UCI and collaborative studies conducted within this section at the Toxic Hazards Research Unit (THRU) at Wright-Patterson Air Force Base, Ohio, during the contract period 1 July 1982 through 30 June 1983. During this period, T.T. Crocker, M.D., was Principal Investigator for the contract. Ronald E. Rasmussen, Ph.D., directed the studies at UCI. Technical personnel at UCI included Staff Research Associates Jean Anderson, Marcia Witte, and Mary Hawley, and Research Assistant Arthur Fong. At the THRU, E.R. Kinkead was Study Director, and R. Scott Bowers was Research Associate. Technical Monitor for the Air Force was M.K. Pinkerton, AFAMRL/THT, Wright-Patterson Air Force Base, Ohio.

During this report period, studies have continued with an animal model tumor system in which lung carcinomas are induced in the rat by intratracheal inoculation with a polycyclic hydrocarbon carcinogen, 3-methylcholanthrene. Factors studied have included cytogenetic effects in lung cells, enzyme induction in lung and liver, and dose-response relationships between carcinogen and the appearance of malignancy.

The goal of the studies is to define some of the biologic alterations that occur during tumorigenesis in order to provide information on which changes are specifically associated with tumor initiation and growth. With this information, the biologic effects of test materials may be more readily evaluated with respect to their carcinogenic potential.

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Statement of the Problem

The process of carcinogenesis requires several steps which, at least conceptually, can be considered separately. The first is the initiation event, which is likely a discrete chemical or physical change at the molecular level involving possibly the genetic material, DNA, or other regulatory biomolecules. Secondly, the cell in which initiation occurs must replicate and express the cancerous phenotype. The growth of a tumor often elicits an immune response as the body attempts to eliminate the "foreign" cells.

In this project the experimental studies have focussed on events surrounding the early stages in carcinogenesis, and the cellular changes that occur in the lung up to the time of tumor appearance. The aim of the studies is to define which of the several observed effects of carcinogen exposure are peculiar to the process of carcinogenesis. This knowledge may aid in the interpretation of effect of materials whose carcinogenic potential is not known.

SPECIFIC AIMS

Using a rat lung tumor model in which squamous cell carcinomas (SCCs) are induced by intratracheal inoculation with a potent carcinogen (3-methylcholanthrene, MCA) the following carcinogenesis-related processes have been studied:

1. The enzymatic activity in the lung and liver which is active in conversion of polynuclear hydrocarbon carcinogens to their carcinogenic forms;

2. Chromosomal damage in lung cells of MCA-treated rats during carcinogen treatment and at later periods up to the time of tumor appearance;

3. The dose-response relationships between MCA treatment and tumor formation;

4. Carcinogen metabolism by specific lung cells in vitro.

5. Cell proliferation in the lungs of MCA treated rats as indicated by DNA synthesis; and

6. Effects on tumorigenesis in this model of combined exposure to MCA and asbestos.

In order to gain additional information on the role of various lung cells in metabolism of carcinogens, studies have been initiated in the mouse to examine the effect of naphthalene and selected derivatives on carcinogen metabolism. The aims of these studies are:

1. To determine the cytotoxic effects of naphthalene (N), 1-nitronaphthalene (1-NN), and 2-nitronaphthalene (2-NN) in the mouse lung; and

2. To measure the levels of carcinogen metabolizing enzymes in lung and liver of treated mice.

MATERIALS AND METHODS

Lung Tumor Induction in the Rat

Squamous cell carcinomas were induced in male Fischer 344 rats (Charles River Labs) by the intratracheal administration of a suspension of MCA crystals in gel-saline (0.2% gelatin in

0.9% NaCl) following the method described by Schreiber et al., 1972. MCA from Sigma Chemical Company (St. Louis, MO) was recrystallized from benzene and ground briefly in a mortar and pestle to provide crystals with a very wide size range from a few micrometers up to several hundred micrometers in length. The rats were anesthetized either with Halothane (at the THRU) or with methoxyflurane (at UCI) and the MCA suspension in 0.1 ml introduced into the lungs via the trachea at the level of the bifurcation, using a Teflon cannula attached to a 0.5 ml glass syringe. In the tumorigenesis studies, rats were inoculated 5 times at biweekly intervals.

Lungs were prepared for examination by inflation fixation via the trachea with buffered 10% formalin, and paraffin sections of the whole lung prepared and stained with hematoxylin and eosin by conventional methods.

In the experiments now in progress, in which rats were treated with both MCA and asbestos, the two materials were given alternately at weekly intervals. In all experiments, control rats were given the gel-saline vehicle at corresponding times.

The asbestos used for inoculation was prepared from a UICC standard sample of chrysotile. Approximately 3 g of the asbestos was shaken repeatedly with glass beads in 1 liter of distilled water and allowed to stand at room temperature for one hour. The larger fibers settled rapidly, leaving the small fibers in suspension. Examination of the latter by light microscopy showed that more than 90% were less than 20 μm in length. The fine fibers were collected by filtration and resuspended in gel-saline for inoculation into the rats. These fine fibers were considered to represent the type which would be inhaled into the deep lung if airborne.

Measurement of Carcinogen Metabolism in Rat Tissues

Methods for measurement of the conversion of ^{3}H-benzo(a)pyrene (^{3}H-BaP) and ^{3}H-MCA to hydroxylated metabolites by microsomal preparations from various tissues have been published (Ramussen and Wang, 1974; Rasmussen, 1982). Briefly, the freshly removed tissues are homogenized in ice cold 1.15% KCl and the microsomal fraction separated by centrifugation. The microsomes are suspended in buffer and incubated in vitro with the labeled carcinogen. Hydroxylated metabolites are extracted from the incubation mixtures with ethyl acetate and isolated by thin-layer chromatography (TLC). The metabolites are quantified by radioactivity measurements using a TLC plate scanner and scintillation counting. Metabolites are identified using authentic derivatives obtained from the National Cancer Institute.

Distribution of MCA Following Intratracheal Inoculation

MCA crystals labeled with ^{14}C were prepared by mixing ^{14}C-MCA (Amersham/Searle) with unlabeled MCA to give a specific radioactivity of 1 μCi/mg. The mixture was dissolved in benzene, and recrystallized by evaporation of the solvent. The crystals were prepared for intratracheal inoculation as above for unlabeled MCA, and rats were inoculated with 0.1 ml of the gel saline suspension for a total dose of 1.0 mg. Sample rats were killed up to 6 weeks post inoculation. The lungs were fixed by inflation with 10% buffered formalin and frozen sections cut for autoradiography. The dried sections were coated with Kodak NTB-2 emulsion and developed after 7 days with Kodak D-19. Additional lung samples were air dried and radioactivity determined by scintillation counting.

Preparation of Lung Cell Cultures for Chromosomal Analysis

The methods used for culturing lung cells were described in detail in the annual report for 1982 (Rasmussen, 1982). In brief, the lungs were inflated with a mixture of thermolysin and collagenase in order to enzymatically disrupt the lung (Frazier et al., 1975). The freed cells were collected by centrifugation and cultured in plastic flasks with Waymouth's 752/1 medium supplemented with 10% fetal bovine serum. Sister chromatid exchanges (SCEs) were visualized using essentially the method of Perry and Wolff (1974). At 24 hours after establishing the cultures 5-bromodeoxyuridine was added to a concentration of 10^{-5} M and incubation continued for an additional 48 hours. Metaphase cells were accumulated by adding colcemid for a period of 3 hours, at which time the cells were collected, fixed, spread on microscope slides and stained with Hoechst 33258 dye and Giemsa blood stain (Latt et al., 1982).

Isolation of Lung Cells for Carcinogen Metabolism Studies

Following perfusion with heparinized normal saline via the pulmonary artery, alveolar macrophages were removed and isolated by pulmonary lavage 5 times with 12 ml of HEPES-buffered balanced saline solution (HpBS). An enzyme solution of 0.1% Protease type I (Sigma Chemical Company) in Hank's balanced salt (HBS) containing thermolysin (Calbiochem, 1500 units/ml), 0.002 M $CaCl_2$, and 1.0% bovine serum albumin (BSA) was instilled into the lung via the trachea and the trachea was then tied off. The lungs were removed from the thorax and incubated in a beaker of HBS at room temperature for 30 min. The lungs were then transferred to a petri dish and minced with scissors. The pieces and mincing solution were transferred to a trypsinizing flask. Additional enzyme solution containing DNAase I (Sigma) and colloidal barium sulfate were added to the flask to a final volume of 100 ml (final concentration of 0.01% DNAase I and 0.01% barium sulfate). Incubation was continued for 30 more minutes. At the end of this period, the enzyme solution containing freed lung cells was removed and the remaining lung tissue was subjected to a second round of enzymatic dispersion (protease and thermolysin for the first 30 min and DNAase I and barium sulfate added for the second 30 min). Cells from the two cycles of dispersion were pooled, washed free of enzyme solution, and resuspended in 10 ml of Waymouth's MB752/1 medium.

Individual cell types were isolated by isopyknic discontinuous density gradient centrifugation (Kikkawa and Yoneda, 1974). Pooled lung cells were layered over a discontinuous density gradient of Ficoll in Waymouth's medium which had the following densities: 1.040 (7 ml), 1.080 (7 ml), 1.100 (14 ml). Centrifugation was performed at 4°C at 350 x g for 20 min.

The cells recovered from the zone above density 1.040 contained approximately 80% type II cells. The cells from the zone above density 1.080 were subjected to a two polymer aqueous phase system composed of 5.0% dextran T 500 and 3.8% polyethylene glycol 6000 in 0.15 M sodium phosphate buffer to further purify the Clara cells (Devereux and Fouts, 1980). A final purity of only 20% Clara cells was achieved from this dense cell layer. Alveolar type II cells were identified using light microscopy by a modified Papanicolou stain (Kikkawa and Yoneda, 1974). Clara cells were identified using a light microscope by a nitroblue tetrazolium method (Devereux and Fouts, 1980). Cell viability was measured by trypan and blue dye exclusion. A viability rate of about 85% was found. The total cells recovered were 4×10^6 type II cells, 2×10^6 dense lung cells, and 1×10^6 alveolar macrophages.

The ability of isolated lung cells to metabolize ^{3}H-BaP was measured by the method described by Rasmussen and Wang (1974). The isolated cells were incubated in 2.0 ml of

Waymouth's medium containing 10% fetal bovine serum, 10 μg/ml gentamicin sulfate, 1.2 mM NADPH, 10 μCi/ml of ^{3}H-BaP (specific activity, 17.4 Ci/mmole) at 37°C for 24 hr. The incubation was stopped by the addition of 4 ml of ethyl acetate. The cells and growth medium were extracted twice with ethyl acetate and the ethyl acetate soluble ^{3}H-BaP and its metabolites were separated by thin-layer chromatography and quantified by measuring the radioactivity in a scintillation spectrometer.

Measurement of DNA Synthesis in Lung Tissue

The methods for this assay have been reported in detail previously (Rasmussen, 1981). Briefly, lungs were inflated in situ with warm melted agar dissolved in a balanced salt solution, removed from the rat, and chilled in ice cold salt solution, and slices of approximately 1 mm thickness cut and incubated with ^{3}H-thymidine (^{3}H-dThd, 5 μCi/ml, 50-60 Ci/mmole) for 1 hour. The specific radioactivity of the DNA was measured by a chemical extraction method and scintillation counting (Scott et al., 1956). This assay was used as an indicator of cell proliferation in lung following MCA treatment.

Treatment of Mice with Naphthalene and its Derivatives

Mice of the Swiss Webster strain (Charles River Laboratory) were treated by ip injection of peanut oil solutions of naphthalene and the nitro derivatives.

Preparation of tissue microsomes, and tissue sections were done as in the case of the rats described above.

EXPERIMENTAL RESULTS

Induction of Lung Tumors in Rats with MCA

A dose-response experiment for the induction of lung tumors in rats by intratracheal MCA was completed in October 1982. The results (Table 1 and Figure 1) showed a marked dose dependence. All rats given 5 biweekly inoculations with 5 mg MCA developed malignant tumors which were identified as squamous cell carcinomas (SCC). At a dose of 5× 1.0 mg MCA, 12/25 rats were found at sacrifice to have malignant SCC and several of the remainder were found with adenomatous hyperplastic lesions, which may be considered to be premalignant. At lower doses of 5× 0.5 mg and 5× 0.25 mg MCA, 4/25 and 2/25 rats, respectively, were found with malignant tumors. No tumors were found in rats treated with 5× 0.1 mg MCA or the 0.2% gelatin: 0.9% NaCl vehicle. The pathology of these tumors was established by Dr. R.L. Bruner at the THRU, and confirmed by Dr. M. Reesal at UCI.

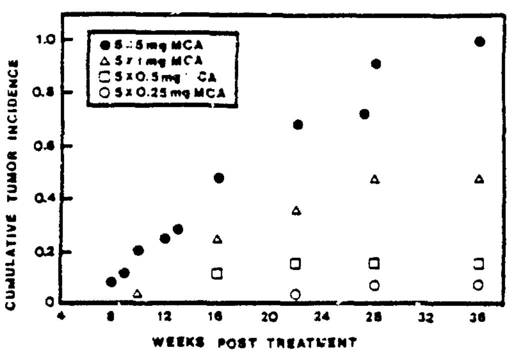

Figure 1. Cumulative Lung Tumor Incidence in MCA-Treated Rats.

Table 1

Lung Tumor Incidence in MCA-Treated Rats

Rats were inoculated intratracheally biweekly with MCA at the indicated doses. At the indicated times, sample groups were sacrificed and examined for lung tumors. All tumors indicated in this table were squamous cell carcinomas.

Weeks Post-Treatment	Vehicle Controls	Rats with Tumor/Rats Killed or Found Dead			
		5 x 0.25 mg	5 x 0.5 mg	5 x 1.0 mg	5 x 5.0 mg
8	-	-	-	-	2/2
9	-	-	-	-	1/1
10	0/5	0/5	0/5	1/5	2/2
12	-	-	-	-	1/1
13	-	-	-	-	1/1
16	0/5	0/5	3/5	5/5	5/5
22	0/5	1/5	1/5	3/5	5/5
27	-	-	-	-	1/1
28	0/5	1/5	1/5	3/5	5/5
36	0/5	0/5	0/5	0/5	2/2
Total	0/25	2/25	4/25	12/25	25/25

Metabolism of ^{3}H-BaP by Lung and Liver Microsomes from MCA-Treated Rats

After a single intratracheal inoculation with 1.0 mg MCA the enzyme activity in lung microsomes toward ^{3}H-BaP remained above controls for at least 6 weeks. With liver microsomes, however, the activity had returned to control levels by 3 weeks posttreatment (Rasmussen, 1982). To test the effect of repeated inoculation with MCA, groups of rats were inoculated with either 0.1 mg, 1.0 mg MCA or 0.1 ml of the vehicle at biweekly intervals. At 48 hr after the first treatment and at selected intervals later, sample rats were killed and the metabolism of ^{3}H-BaP by lung and liver microsomes measured. The treatment and sacrifice schedule is shown in Table 2, and the results shown in Table 3 and in Figures 2 and 3. The enzymatic activity of the lung microsomes was increased to about the same extent at both doses of MCA, and remained at approximately the same level throughout the course of the treatment. After the final treatment, the activity declined with time until at 8 weeks the enzyme activity was not different from that of the vehicle controls. This pattern was essentially the same as that seen after a single treatment with MCA, i.e., repeated treatment did not result in an extension of the time when enhanced enzyme activity was present. With liver microsomes the pattern was also similar to that seen after a single inoculation with MCA. During the treatment enzyme activity was elevated, with that seen with microsomes from the higher dose groups always greater than controls. The activity with microsomes from the lower dose groups was greater than controls only in that group sampled at 48 hr after the first treatment. At 3 weeks after the last treatment liver microsome activity was similar in all groups.

The above results may be directly related to the persistence of MCA in the lungs of the treated rats. Experiments were done to measure MCA clearance in which rats were inoculated with ^{14}C-MCA crystals prepared in the same way as for the enzyme and tumorigenesis studies. Groups of rats were inoculated with the ^{14}C-MCA and sample rats killed at

Table 2

Treatment and Sacrifice Schedule for Repetitive Dose Study with MCA

Date	Action	Time Post-Treatment	No. of Animals	Untreated	Vehicle	0.1 mg MCA	1.0 mg MCA
6-1-82	1st Inoc.	—	105	—	35	35	35
6-2-82	1st Inoc.	—	15	—	5	5	5
6-4-82	Sacrifice	48 hr	20	5	5	5	5
6-8-82	Sacrifice	1 wk	15	—	5	5	5
6-15-82	2nd Inoc.	2 wk	72	—	24	24	24
6-22-82	Sacrifice	3 wk	12	—	4	4	4
6-29-82	3rd Inoc.	4 wk	60	—	20	20	20
7-6-82	Sacrifice	5 wk	12	—	4	4	4
7-13-82	4th Inoc.	6 wk	48	—	16	16	16
7-20-82	Sacrifice	7 wk	12	—	4	4	4
7-27-82	5th Inoc.	8 wk	36	—	12	12	12
8-17-82	Sacrifice	11 wk	12	—	4	4	4
8-31-82	Sacrifice	13 wk	12	—	4	4	4
9-21-82	Sacrifice	16 wk	16	4	4	4	4

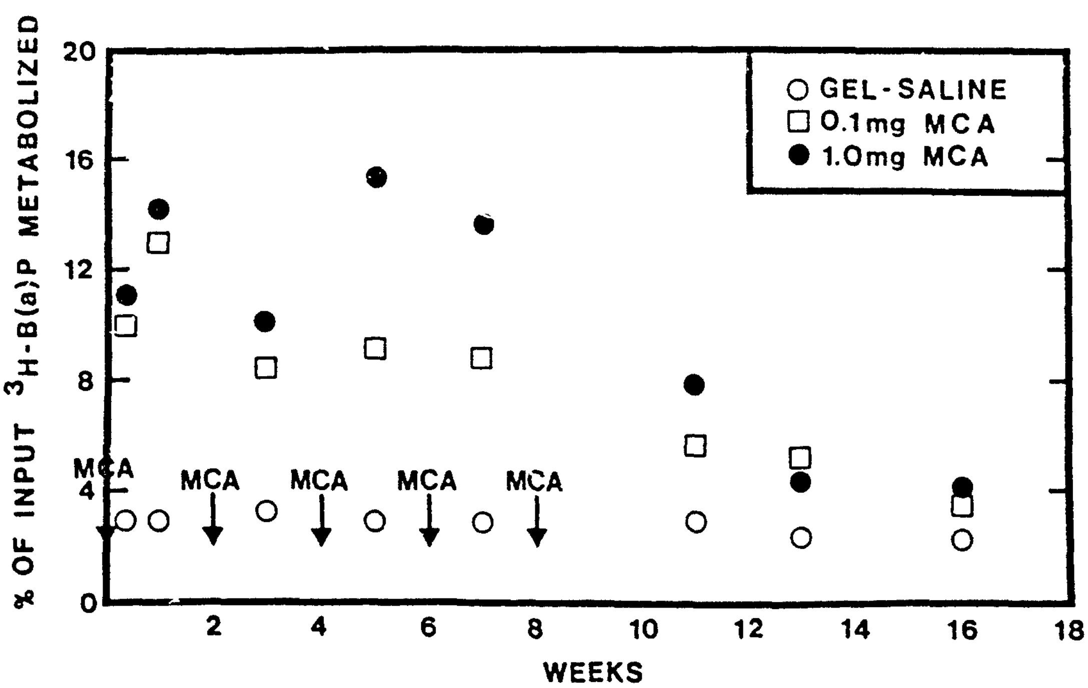

Figure 2. Metabolism of ^{3}H-BaP by Lung Microsomes.

Table 3

^{3}H-BaP Metabolism by Lung and Liver Microsomes from Rats Treated Repeatedly with MCA

Values are the percentage of ^{3}H-BaP in the incubation mixture converted to hydroxylated products during a 10 min incubation, and are the average of duplicate samples. Blank values have not been subtracted.

Time After/ Treatment	Lung Microsomes	Liver Microsomes
48 hr/Untreated	4.14	14.0
48 hr/Gel-Saline	2.72	15.1
48 hr/0.1 mg MCA	10.1	25.3
48 hr/1.0 mg MCA	11.0	68.8
48 hr Blank	2.79	
1 wk/1 x Gel-Saline	2.76	17.9
1 wk/1 x 0.1 mg MCA	13.2	14.4
1 wk/1 x 1.0 mg MCA	15.2	54.1
1 wk Blank	1.77	
3 wk/2 x Gel-Saline	3.33	15.7
3 wk/2 x 0.1 mg MCA	8.30	14.9
3 wk/2 x 1.0 mg MCA	10.4	47.9
3 wk Blank	2.32	
5 wk/3 x Gel-Saline	2.92	16.04
5 wk/3 x 0.1 mg MCA	9.19	17.06
5 wk/3 x 1.0 mg MCA	15.37	57.80
5 wk Blank	2.06	
7 wk/4 x Gel-Saline	3.09	16.65
7 wk/4 x 0.1 mg MCA	8.83	16.98
7 wk/4 x 1.0 mg MCA	13.70	72.07
7 wk Blank	2.45	
11 wk/5 x Gel Saline	3.09	14.5
11 wk/5 x 0.1 mg MCA	5.76	12.6
11 wk/5 x 1.0 mg MCA	7.77	19.6
11 wk/Blank	3.38	
13 wk/5 x Gel Saline	2.44	14.5
13 wk/5 x 0.1 mg MCA	5.21	14.4
13 wk/5 x 1.0 mg MCA	4.52	14.4
13 wk/Blank	3.25	
16 wk/5 x Gel Saline	2.41	12.7
16 wk/5 x 0.1 mg MCA	3.56	13.6
16 wk/5 x 1.0 mg MCA	3.63	12.8
16 wk/Blank	4.12	

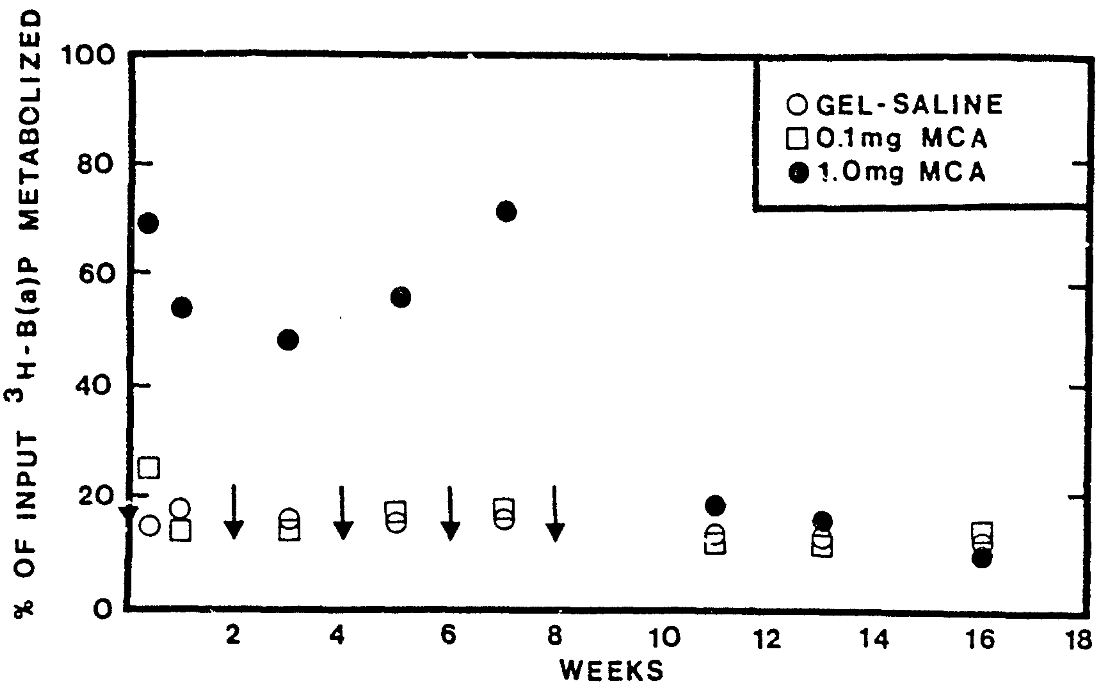

Figure 3. Metabolism of ^{3}H-BaP by Liver Microsomes.

intervals. Labeled MCA associated with the lung was measured by scintillation counting of tissue digests, and by autoradiography of frozen sections of formalin-fixed lungs. Radioactivity measurements (Table 4) indicated that by 6 weeks following a single inoculation with 1.0 mg ^{14}C-MCA, nearly all of the MCA had been cleared from the lung. Autoradiographs were analyzed by counting centers of radioactivity as indicated by clusters of silver grains in full cross-sections of the rat lungs. These centers of radioactivity were the result of local deposition of large MCA crystals. The data (Table 5) indicated that ^{14}C-MCA was cleared with time, but the experiment did not shed any light on the mechanism of clearance.

Table 4

^{14}C-MCA Clearance from Rat Lung

Values are ^{14}C-dpm per mg protein and are the average of single determinations on each of 2 rats.

Time	^{14}C-dpm/mg Protein
2 hr	16,157
7 days	613
6 weeks	42

Table 5

Clearance of ^{14}C-MCA from Rat Lung

Autoradiographs of lung sections were scanned and the total number of clusters of silver grains for each lung were counted. Two or three sections were counted from each rat, and two rats were killed at each sample time.

Time After Treatment	Rat #1	Rat #2
	1.0 mg ^{14}C-MCA	
0	576,419. $\bar{x}$ = 498 ± 111	156,134. $\bar{x}$ = 145 ± 16
48 hr	359,373,663. $\bar{x}$ 465 ± 172	482,536,520. $\bar{x}$ = 513 ± 28
6 day	71,95,118. $\bar{x}$ = 95 ± 24	84,136,101. $\bar{x}$ = 107 ± 27
2 wk	85,78,50. $\bar{x}$ = 71 ± 19	52,61,100. $\bar{x}$ = 71 ± 26
	0.1 mg ^{14}C-MCA	
	Rat #1	**Rat #2**
0	91,73. $\bar{x}$ = 82 ± 13	45,13. $\bar{x}$ = 29 ± 23
48 hr	5,6,7. $\bar{x}$ = 6 ± 1	28,27,28. $\bar{x}$ 28 ± 1
6 day	26,31,18. $\bar{x}$ = 25 ± 7	7,11,14. $\bar{x}$ = 11 ± 4
2 wk	3,3,6. $\bar{x}$ = 4 ± 2	2,0,4. $\bar{x}$ = 2 ± 2

Metabolism of ^{3}H-BaP by Lung Cells

A preliminary study was done to determine whether a difference might exist between different types of lung cells with respect to their ability to metabolize PAH and whether the metabolizing enzymes are inducible. Groups of rats were treated with either an inducer of PAH metabolism, beta-naphthoflavone (BNF) or the corn oil vehicle. After 48 hr, the lungs were removed, enzymatically dispersed, and the cells fractionated using isopyknic gradient centrifugation (as described under Methods). Alveolar macrophages were obtained by lavage prior to the enzyme treatment. A cell fraction containing approximately 80% type II alveolar cells and a fraction containing more dense cells (approx. 20% Clara cells) were incubated for 24 hr with ^{3}H-BaP in nutrient medium. Metabolites were isolated by thin layer chromatography and quantified by scintillation counting. The results (Table 6) indicated an increase in ^{3}H-BaP metabolism in the type II cells and dense cells from the BNF treated rats. The alveolar macrophages from the BNF treated rats showed less activity than the controls. These experiments indicate that cell separation and measurement of enzymatic activities is possible, and that further fractionation of cell groups with the centrifugal elutriator should yield important information regarding the metabolism of PAH by the various cell types.

Metabolism of ^{3}H-MCA by Microsomes from MCA-Treated Rats

Rats were inoculated intratracheally with 1.0 mg MCA and sample groups killed at 48 hr, 1 week, 3 weeks and 6 weeks posttreatment. Microsomes were prepared from lung and liver and incubated with ^{3}H-MCA in the same manner as previously described for ^{3}H-BaP. The total metabolite yields are summarized in Table 7. Enzyme activity in the lung microsomes was above control levels for at least 6 weeks, but had returned to control levels in the liver by 3 weeks posttreatment. Although several metabolites were detected on thin layer

Table 6

Metabolism of ^{3}H-BaP by Isolated Lung Cells

Male Fischer rats were treated with either corn oil (CO) or beta-naphthoflavone (BNF, 80 mg/kg BW). At 48 hr posttreatment, lung cells were isolated and incubated with ^{3}H-BaP for 24 hr at 37°C. The blank values from incubations without lung cells have been subtracted from the values for the amount of metabolites produced. Values are femtomoles of metabolites per μg DNA.

Metabolite	Type II CO	Type II BNF	Dense Cells CO	Dense Cells BNF	Macrophages CO	Macrophages BNF
Origin	52.2	186.0	95.5	152.8	75.6	36.7
9,10-Diol	73.6	236.0	68.3	252.0	39.9	14.2
7,8-Diol	170.6	288.8	126.1	197.4	136.7	34.0
4,5-Diol	211.7	341.1	131.4	189.6	279.8	122.1
Mono-OH BaP	208.1	270.7	123.6	237.4	284.9	124.7
Diones	1505.7	3574.6	1033.6	1049.5	1659.8	1091.4

Table 7

^{3}H-MCA Metabolism by Lung and Liver Microsomes from MCA-Treated Rats

Values are the percentage of input ^{3}H-MCA converted to polar metabolites during the incubation period, and are the mean of triplicate samples ± S.D. Blank values have not been subtracted. Blank incubation mixtures contained heat-inactivated microsomes.

Treatment/ Time Posttreatment	Lung Microsomes	Liver Microsomes
MCA/48 hr	17.58 ± 0.56	47.30 ± 3.35
Vehicle/48 hr	5.47 ± 1.01	24.07 ± 1.29
Untreated/48 hr	4.38 ± 1.00	25.90 ± 1.01
Blank	1.97 ± 0.37	2.75 ± 0.39
MCA/1 week	14.32 ± 0.81	25.07 ± 0.46
Vehicle/1 week	3.05 ± 0.15	17.08 ± 0.84
Blank	2.02 ± 0.23	2.04 ± 0.17
MCA/3 wk	8.63 ± 0.28	23.34 ± 4.93
Vehicle/3 wk	3.20 ± 0.08	18.22 ± 0.49
Blank/3 wk	2.14 ± 0.22	2.41 ± 0.41
MCA/6 wk	4.20 ± 0.71	20.12 ± 1.02
Vehicle/6 wk	2.93 ± 0.12	20.33 ± 1.28
Untreated/6 wk	2.66 ± 0.28	19.12 ± 0.74
Blank/6 wk	2.12 ± 0.11	2.33 ± 0.23

chromatography, they were not identifiable because of the lack of authentic standards. Figure 4 shows radioscanner tracings of the TLC plates which indicate the formation of at

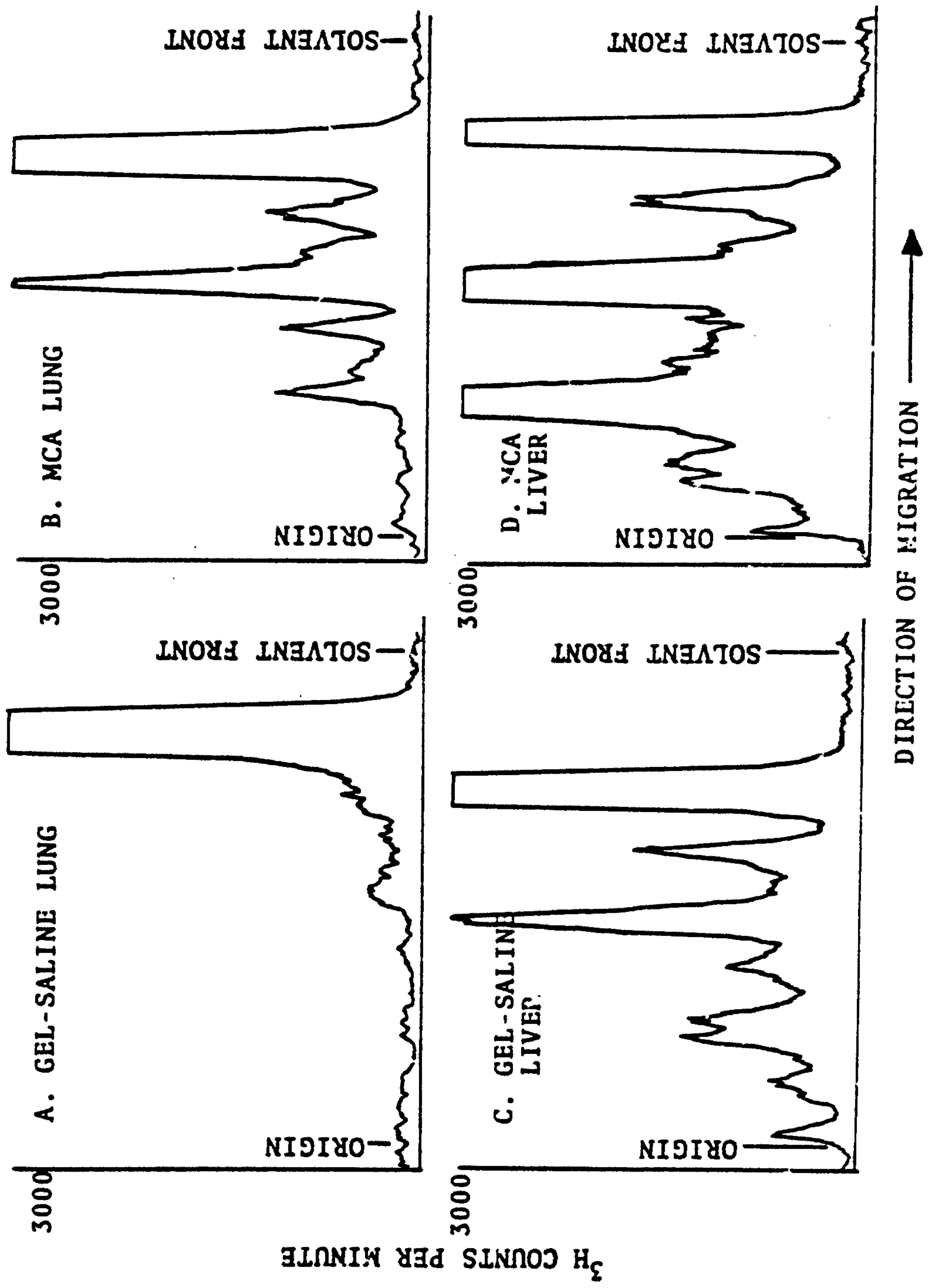

Figure 4. Tracings of Radioscans of TLC Plates Showing Metabolites of ^{3}H-MCA.

least 6 metabolites of MCA. Quantitatively, the total metabolism of ^{3}H-MCA was similar to that found when ^{3}H-BaP was the substrate.

SCE Incidence in Lung Cells and Lymphocytes from MCA-Treated Rats

Two questions were addressed in these experiments. The first was whether MCA treatment induced DNA damage in lung cells which could be detected as an increased incidence of SCEs when the cells were cultured in vitro. The second question was whether intratracheal inoculation with MCA caused an increase in SCEs in circulating lymphocytes.

Table 8 shows data on SCE incidence in rat lung cell cultures prepared up to 8 weeks following a single inoculation with either 0.1 mg or 1.0 mg MCA. At the higher dose SCE frequency declined with time, but was higher than controls through the 8th week posttreatment. Lung cells from rats given the lower dose showed an SCE frequency which was statistically significantly higher than the controls at the 4th and 8th weeks, but the differences were small.

Table 8

Incidence of SCEs in Primary Cultures of Lung Cells from MCA-Treated Rats

Values are SCEs per chromosome ± S.D. The number of cells scored is in parentheses. Metaphases containing 30 or more chromosomes were scored.

Time Post-Treatment	Vehicle	0.1 mg MCA	1.0 mg MCA
48 hr	0.611 ± 0.18 (71)	No data	1.248 ± 0.23 (73)[a]
2 wk	0.683 ± 0.25 (99)	0.731 ± 0.18 (22)[b]	0.957 ± 0.31 (41)[a]
4 wk	0.513 ± 0.15 (98)	0.655 ± 0.20 (98)[c]	0.686 ± 0.21 (89)[a]
6 wk	0.578 ± 0.20 (100)	0.624 ± 0.22 (66)[b]	0.907 ± 0.28 (58)[a]
8 wk	0.529 ± 0.18 (97)	0.600 ± 0.22 (100)[c]	0.735 ± 0.39 (35)[d]

[a]Significantly greater than vehicle control ($p < 0.0005$).
[b]Not significantly different from vehicle control.
[c]Significantly greater than control ($p < 0.01$, $t = 2.448$).
[d]Significantly greater than control ($p < 0.005$, $t = 7.984$).

To test for possible induction of SCEs by MCA in peripheral lymphocytes, rats were inoculated intratracheally with 2.5 mg MCA. Two days later lymphocytes were isolated from peripheral blood and cultured for SCE analysis. The results from 2 such experiments are shown in Table 9. Although slightly higher SCE frequency was seen in one experiment in the lymphocytes from the MCA treated rats, the difference was small, and may not be indicative of an actual effect of MCA.

A further study was done using lymphocyte cultures prepared from peripheral blood and spleens of rats treated 5 times with MCA and/or asbestos, using a treatment schedule expected to produce lung tumors. Twenty-four hours after the last intratracheal treatment, 3 rats from each group were sacrificed and cultures prepared from spleen and blood lymphocytes for SCE analysis as described previously. The results are summarized in Table 10. Although statistical differences were seen in some cases, they were marginal and based on previous

experience should be accepted with caution. The data suggest that repeated MCA or ASB treatments which can be expected to produce lung tumors do not result in marked elevation of SCEs in peripheral blood or spleen lymphocytes.

Table 9

SCE Incidence in Peripheral Lymphocytes from MCA-Treated Rats

Values are SCEs/chromosome ± S.D. Metaphases with 33 or more chromosomes were scored, and 100 metaphases were scored for each value shown.

Treatment	SCEs/Chromosome
Control (3 month old rats)	0.217 ± 0.086
MCA (3 month old rats)	0.248 ± 0.100[a]
Control (5 month old rats)	0.331 ± 0.164
MCA (5 month old rats)	0.294 ± 0.106[b]

[a]Significantly different from control; t = 3.55.
[b]Not significantly different from control; t = 1.93.

Table 10

SCE Incidence in Blood and Spleen Lymphocytes from Rats Treated with MCA or ASB[a]

Lymphocytes	Treatment	SCEs/Chromosome	"t" vs Gel Saline
Blood	Gel Saline	0.315 ± 0.135	—
Blood	MCA	0.248 ± 0.104	3.93[b]
Blood	ASB	0.336 ± 0.115	1.18
Blood	MCA + ASB	0.365 ± 0.129	2.68
Spleen	Gel Saline	0.428 ± 0.189	—
Spleen	MCA	0.396 ± 0.168	1.26
Spleen	ASB	0.463 ± 0.164	1.40
Spleen	MCA + ASB	0.527 ± 0.221	3.40[c]

[a]Values are SCEs per chromosome ± S.D. and are based on counts of 100 cells containing 35 or more chromosomes.

[b]Different from Gel Saline at $p < 0.001$ (2 tailed "t" test).

[c]Different from Gel Saline at $p < 0.01$ (2 tailed "t" test).

Experiments in which MCA was added to cultures of lung cells from untreated rats indicated that such cells were susceptible to the clastogenic effects of MCA, and that as little as 1 μg MCA per ml of culture medium was sufficient to increase the SCE frequency very significantly. Therefore, even a small amount of MCA remaining in the lung and/or carried over during the preparation of the lung cell cultures would be expected to increase the SCE frequency (Rasmussen, 1982).

Considering these results together with those on MCA clearance from the lung (Tables 4 and 5) the most likely conclusion is that the production of SCEs in lung cells is dependent on the continued presence of MCA in the lung. The possibility that repeated inoculation with MCA might produce a more persistent increase in SCE frequency in lung cells was examined in cell cultures prepared from rats treated five times with either 0.1 mg or 1.0 mg MCA. The results (Table 11) indicated that even repeated treatment did not lead to significant elevation of SCE frequency that persisted long past treatment.

Table 11

SCE Incidence in Lung Cells after 5 Biweekly Treatments with MCA

Cultures were prepared 3 weeks following the last inoculation with MCA. Values are as in Table 8.

Treatment	SCEs/Chromosome (n)
Gel-Saline Control	0.557 ± 0.215 (39)
5 × 0.1 mg MCA	0.672 ± 0.241 (43)*
5 × 1.0 mg MCA	0.561 ± 0.218 (22)

*Greater than control ($p < 0.01$, $t = 2.489$).

Cellular Proliferation in Rat Lung After Intratracheal Inoculation with MCA and/or Asbestos

Previous studies of rat lungs after a single intratracheal inoculation with MCA did not show an increased level of overall DNA replication or any evidence for DNA repair activity. However, the methods employed for DNA repair measurement may not have been sensitive enough since it has been shown in cell culture systems that PAH such as MCA can induce DNA repair activity. During the current year, studies have been done in an attempt to identify foci of cellular proliferation which may be related to further tumor formation.

An experiment was initiated in December 1982 to determine whether the rats would tolerate weekly intratracheal inoculations and also to determine the effects of combined treatment with MCA and asbestos. Four groups of rats (Fischer 344 males, approximately 100 g bw at the beginning of the experiment) were inoculated weekly with either 0.1 ml gel:saline, alternately with 1.0 mg MCA or gel:saline, alternately with 1.0 mg MCA or 0.1 mg chrysotile asbestos, or alternately with 0.1 mg asbestos or gel:saline. After 10 inoculations, rats from each group were injected with ^{3}H-thymidine (^{3}H-dThd, 200 μCi/rat), killed 1 hour later, and the lungs fixed by inflation with buffered formalin. The lungs were sampled according to the recommendations of Dungworth et al. (1976) and paraffin sections prepared for examination and autoradiography.

Sections from 2 rats inoculated with gel:saline only showed no significant lesions, i.e., no hyperplasia, necrosis, pneumonia, tumor, etc. (The sections were examined in consultation with Dr. Michael Reesal). Lungs from 4 rats treated with MCA only showed some changes, mostly focal areas of alveolar cell hyperplasia, and bronchiolar epithelial cell hyperplasia. The lobes most affected were the right caudal lobe and the accessory lobe. A small tumor, appearing to be of bronchiolar epithelial cell origin, was seen in the left lobe of one rat.

Lungs from 4 rats treated with asbestos only showed no tumors, but did show small nodules, possibly early fibrosarcomas. All lobes of all animals showed some changes with focal alveolar cell hyperplasia being most frequent.

Lungs from 5 rats treated alternately with MCA and asbestos showed all of the above changes to a more marked degree, and, in addition, 2 of the rats had SCC. One animal had 3 separate SCCs, each in a different lobe. Four of the 5 had changes which might be considered pre-cancerous, i.e., pronounced bronchiolar epithelial cell hyperplasia and invasion of the alveoli by epithelial cells of the terminal bronchioles. One rat of this group showed a small area of pneumonia in the accessory lobe (PMNs and macrophage in the alveoli, but no bacteria visible).

Autoradiographs were prepared from sections adjacent to those examined as described above. In the normal adult rat lung, the labeling index, which indicates the fraction of cells undergoing replication, is about 0.001, and labeled cells are widely scattered throughout the lung. In the treated animals, concentrations of labeled cells were found in the areas of alveolar and bronchiolar epithelial hyperplasia described above. The fibrotic nodules also showed the presence of labeled cells. Where tumors were present, cellular labeling was very frequent with the labeled cells concentrated around the periphery of the tumor.

These studies suggest that early changes, possibly related to tumor formation, can be found shortly after exposure to carcinogens. Also, the apparent enhancement of tumor formation in the rats receiving both MCA and asbestos suggests a cocarcinogenic action of the two agents. The experiments now underway at the THRU will provide further information on the latter possibility.

Cytotoxic Effects of Nitronaphthalene in the Mouse

Naphthalene has been shown to cause necrosis of the nonciliated bronchiolar cells (Clara cells) in the mouse when the compound is given at relatively high doses (Mahvi et al., 1977). More recently, this loss of Clara cells has been associated with reduced enzyme activity in the lung, in particular the mixed function oxidases that are responsible for metabolism of aromatic hydrocarbons and other drugs (Tong et al., 1981). Under separate support, these effects of naphthalene have been confirmed and extended in this laboratory.

As part of an undergraduate research project during the past year, comparative studies were initiated involving the nitroderivatives of naphthalene, namely 1-nitronaphtalene and 2-nitronaphthalene. A major finding was that the presence of the nitro group, as well as its position, significantly alters the toxicity in the mouse.

The comparative cytotoxic effects of N, 1-nitronaphthalene, and 2-nitronaphthalene were determined by dosing mice with the compounds dissolved in peanut oil, and sacrificing sample animals at intervals posttreatment. Table 12 summarizes the cytotoxic effects found. 1-nitronaphthalene was the most toxic, with no mice surviving at a dose of 2 mmoles/kg, and naphthalene was next most toxic, followed by 2-nitronaphthalene. However, 2-nitronaphthalene at 2 mmoles/kg did produce periportal necrosis in the liver, which was not seen with the other compounds.

Enzymatic activity of lung and liver microsomes toward ^{3}H-BaP was measured as described in the Methods section. The results of 2 experiments are shown in Table 13. In agreement with previous results, the activity of the lung microsomes from naphthalene-treated mice was lower than that of the controls. However, the activity of microsomes from mice treated with 1-nitronaphthalene was slightly lower in one experiment, and slightly higher in the other, even though there was toxicity to the Clara cells in the lung, as confirmed by examination of tissue samples. Of most interest was the effect with 2-nitronaphthalene. The activity of the lung enzymes was increased about 2-fold over the control level in both

Table 12

Cytotoxic Effects in Mouse Lung Produced by Naphthalene 1-Nitronaphthalene and 2-Nitronaphthalene[a]

Time after treatment	Extent of damage					
	N (2 mmole/kg)	Peanut Oil	(0.28)	(0.56)	(1.0)	(2.0)
		1-NN (mmole/kg)				
24 hrs	3.9	1.0	1.7	1.9	3.6	all died
72 hrs	2.6	0	1.2	0.7	1.3	all died
7 days	1.6	0	1.2	1.2	1.1	all died
		2-NN (mmole/kg)				
24 hrs	3.9	1.0	1.2	1.4	0.9	2.1
72 hrs	2.6	0	1.3	0.6	0.5	0.6
7 days	1.6	0	1.3	1.5	1.0	0.7

[a]The values represent damage scored on an arbitrary scale of 0–5 as follows: 0, no significant effects; 1+, swelling of Clara cells and some sloughing in terminal bronchioles but not in bronchi or trachea; 2+, sloughed Clara cells in bronchioles but minimal effects in bronchi and trachea; 3+, sloughed Clara cells throughout airways, but ciliated cells intact; 4+, sloughed Clara and ciliated cells in bronchioles; 5+, sloughed Clara and ciliated cells throughout all airways. The values are the average of 2 mice for each sample time. Slides were scored without prior knowledge of the treatment.

experiments, suggesting that this compound had an enzyme-inducing effect. By contrast, none of the compounds had any significant effect on the activity of liver enzymes.

While there are many xenobiotics and drugs that induce mixed function oxidases in both liver and lung, and several that are active only in the liver (e.g., phenobarbital), compounds that are specific inducers in the lung are rare. A brief survey of standard texts in toxicology and consultation with colleagues at UCI revealed no examples of compounds that are specific inducers for lung mixed function oxidases. This unusual specificity of 2-nitronaphthalene toward the lung gains further importance from the fact that the nitronaphthalenes occur as air pollutants as the result of fossil fuel combustion, especially in diesel engines. Modification of lung enzymes by these materials may have important implications for human health.

Table 13

Metabolism of ^{3}H-BaP by Lung and Liver Microsomes from Mice Treated with Naphthalene, 1-Nitronaphthalene or 2-Nitronaphthalene

Values are pmoles of metabolite per mg protein during a 30 min incubation period, and are the average of 3 samples ± S.D.

Metabolite	Peanut Oil	N (2 mmoles/kg)	1-NN (1 mmoles/kg)	2-NN (2 mmoles/kg)
Experiment 1:				
Lung Microsomes				
TLC Origin	25 ± 5	23 ± 12	11 ± 12	23 ± 1
9,10-Diol	24 ± 3	15 ± 2	25 ± 4	59 ± 1
7,8-Diol	35 ± 3	25 ± 1	43 ± 3	84 ± 4
4,5-Diol	28 ± 3	17 ± 2	27 ± 6	55 ± 4
Phenols	115 ± 6	72 ± 4	156 ± 17	237 ± 11
Liver Microsomes				
TLC Origin	1180 ± 311	1681 ± 207	1437 ± 60	992 ± 47
9,10-Diol	513 ± 71	635 ± 22	511 ± 63	388 ± 49
7,8-Diol	986 ± 292	1212 ± 274	1331 ± 235	879 ± 35
4,5-Diol	1119 ± 47	1201 ± 65	848 ± 201	1003 ± 67
Phenols	1437 ± 232	1242 ± 83	1392 ± 72	1680 ± 66
Experiment 2:				
Lung Microsomes				
TLC Origin	21 ± 10	11 ± 2	6 ± 5	18 ± 5
9,10-Diol	18 ± 2	12 ± 1	11 ± 0	54 ± 2
7,8-Diol	28 ± 2	19 ± 1	21 ± 1	71 ± 1
4,5-Diol	23 ± 2	11 ± 1	12 ± 1	50 ± 2
Phenols	119 ± 10	67 ± 2	82 ± 14	225 ± 18
Liver Microsomes				
TLC Origin	1433 ± 202	1370 ± 224	1108 ± 61	1236 ± 76
9,10-Diol	529 ± 33	470 ± 41	409 ± 22	430 ± 40
7,8-Diol	788 ± 66	617 ± 94	562 ± 26	532 ± 69
4,5-Diol	1404 ± 183	1482 ± 99	1189 ± 136	1337 ± 106
Phenols	1404 ± 17	1201 ± 123	1237 ± 110	1348 ± 33

DISCUSSION AND CONCLUSION

The tumorigenesis studies in rats conducted at the THRU with MCA showed a strong dose dependence for the initiation of SCCs in the lung. Although the numbers of animals used were much too small to define a precise dose-response curve or threshold, sufficient information was obtained to be able to specify doses that would give a low yield of tumors or tumors in 100% of the animals. These findings have already been applied in the design of an experiment, now in progress at the THRU, to investigate the cocarcinogenic effects of simultaneous treatment with MCA and asbestos. In this experiment, rats have been treated with a dose of MCA expected to give a very low tumor yield, and also treated with chrysotile asbestos, both materials given intratracheally. A cocarcinogenic effect of asbestos would be seen as an

increase in the yield of lung tumors over that seen with MCA alone. The results of this study will appear in the next annual report.

Studies of the clearance of MCA from the rat lung indicated that the compound was nearly completely cleared from the lung within a few weeks. The clearance was paralleled by the SCE frequency in lung cell cultures prepared from treated rats. These results indicate that most DNA damage induced by MCA does not persist, and is likely repaired. Previous work has shown that lung cells have the capacity for DNA repair synthesis following chemically-induced damage to DNA (Rasmussen, 1981). While these experiments do not rule out the possibility that DNA damage persists in those particular cells destined to develop into tumors, they do show that, overall, elevated SCE levels depend on MCA being present during or immediately prior to the SCE assay. It may be concluded from these studies that assay of SCE frequency in vivo with the aim of detecting genotoxic effects of test chemicals should be done simultaneously with or very shortly following chemical exposure.

The use of peripheral lymphocyte cultures for assay of SCE frequency has been used to demonstrate genotoxic effects of some chemicals both in experimental animals and in humans. This approach is attractive since it would allow survey of exposed persons for evidence of exposure to potentially hazardous materials. In the present experiments lymphocyte cultures were prepared from both peripheral blood and spleens of rats after single or multiple treatments with MCA at doses expected to produce lung tumors. In these studies no elevation of SCE frequency was found in the lymphocytes, although lung cells from the same animals did show an increase. Therefore, SCE assay in peripheral lymphocytes may not be a reliable indicator of genotoxic effects of chemicals. The nature of the chemical, route of exposure, and metabolic pathways will all influence genotoxicity.

Measurements of carcinogen metabolism in lung and liver of MCA-treated rats showed that enzyme levels in the lung followed the clearance of MCA from that organ, while enzyme levels in the liver returned to control levels even though significant amounts of MCA remained in the lung. This observation is probably best interpreted as a dosage effect, with concentrations of MCA being generally higher in the lung than in the liver. The results suggest that during the final clearance stages, MCA may be largely metabolized by lung enzymes. The experiments with isolated lung cells demonstrated that ^{3}H-BaP metabolism could be studied in isolated cells and also that it was possible to show induction of enzyme activity in specific cell types. Further experiments with isolated cells are underway and results will appear in future reports.

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